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Evolution of Experimental and Theoretical Determinations of Protein Structure and Protein Folding Pathways

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Physical chemical studies of hydrogen bonding and hydrophobic interactions, and experimental studies of the structure and folding pathways of bovine pancreatic ribonuclease A motivated the development of a theoretical approach to compute protein structure and protein-folding pathways.

1 Introduction

This article traces the development of our experimental and theoretical efforts to gain an understanding of the underlying physics that controls the progression from a newly-synthesized polypeptide chain to the three-dimensional structure of a native biologically-active fibrous or globular protein. Our earliest involvement with this problem was concerned with the influence of hydrogen bonds and hydrophobic interactions on protein structure and reactivity. This work led to our efforts to determine protein structure and folding pathways, first by experimental methods, and subsequently by theoretical methods.

2 Internal Bonding in Proteins

Internal hydrogen bonds influence the observed pKs of ionizable groups¹ and even the reactivity of covalent bonds², e.g., peptide bonds. Figure 1 provides an example of a hydrogen bond between a tyrosyl donor and a glutamate acceptor. The observed pKs of

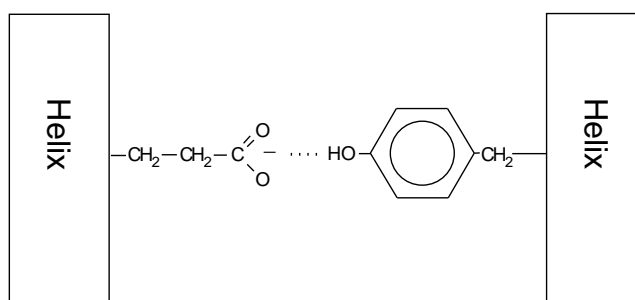


Figure 1. Example of a tyrosyl \cdots glutamate hydrogen bond.

these groups are modified by the free energy to form (or break) such a hydrogen bond. Therefore, in comparison with a non-hydrogen-bonded model compound, the observed pKs of such hydrogen-bonded tyrosyl and glutamate groups will be raised or lowered, respectively. Consequently, such departures of the pKs from those of model compounds are diagnostic for the presence of such hydrogen bonds.

Hydrophobic interactions can provide a nonpolar environment which will also influence the pKs of nearby ionizable groups. A theory³ for the thermodynamics of hydrophobic interactions, based on the structures of liquid water and of aqueous hydrocarbon solutions was presented in 1962, and upgraded⁴ in 2004. By themselves, hydrogen bonds in proteins in water are not very strong because of the necessity to shed water in order to form a hydrogen bond between the polar groups of a protein. However, as illustrated in Figure 2, the presence of nearby nonpolar groups can provide hydrophobic interactions⁵ with the nonpolar parts of residues such as lysine and glutamic acid and also restrict the internal rotational freedom of the ionizable side chains. In addition, nonpolar groups can restrict the access of water to the polar parts of ionizable side chains. Thus, the cooperativity of nonpolar groups and hydrogen bonding of ionizable side chains can strengthen the hydrogen bonds.

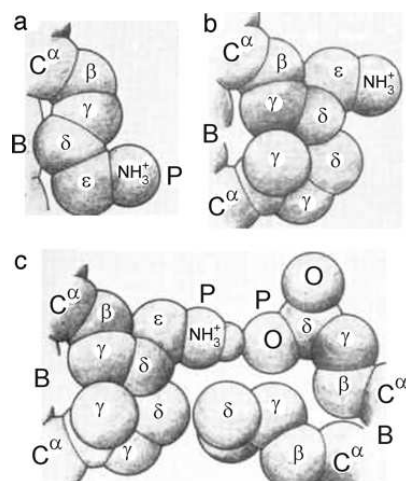


Figure 2. Illustration of various hydrophobic interactions of a polar side chain with its surroundings. B refers to the backbone, and P to the polar head.

3 Location of Hydrogen Bonds in Proteins

Before the advent of X-ray crystallography, NMR, and recombinant DNA methods to determine protein structure, experimental studies to locate hydrogen bonds between ionizable groups, as indicated by the dotted lines in Figure 3, provided distance constraints on the folding of a protein backbone. Such studies, carried out on the 124-residue protein bovine pancreatic ribonuclease A (RNase A), showed that 3 of its 6 tyrosyl residues had

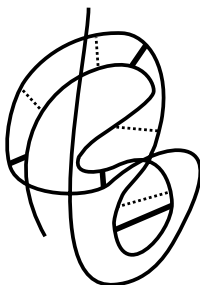


Figure 3. Schematic representation of a protein. Solid and dotted lines represent disulfide bonds and non-covalent interactions, respectively.

abnormally-high⁶ pKs, and that 3 of its 11 carboxyl groups had abnormally-low⁷ pKs. Further, the UV absorption spectrum of tyrosine was perturbed⁸ at low pH where carboxyl groups ionize. This evidence suggested the existence of three tyrosyl \cdots carboxylate hydrogen bonds, and subsequent physical and biochemical experiments⁷ identified one pairing, namely

Tyr25 \cdots Asp14
Tyr92 \cdots Asp38
Tyr97 \cdots Asp83

out of the 19,800 possible ways to pair 3 of 6 Tyr and 3 of 11 carboxyl groups of RNase A. The identification of these 3 interactions was subsequently verified by the X-ray structure. These three non-covalent interactions (dotted lines in Figure 3) and the four disulfide bonds (solid-line crosslinks in Figure 3) provide 7 distance constraints on the folding of the backbone. However, 7 distance constraints are not sufficient to provide an accurate description of the backbone of a 124-residue protein such as RNase A. To determine the backbone structure, as is now done by NMR, many more distance constraints would be required. In fact, it is possible to specify the number of distance constraints required⁹ in order to determine the structure within any desired RMSD from the native structure.

4 Initial Considerations of a Theoretical Approach to Structure Simulation

On the other hand, even 7 known distances could serve as restraints on a potential energy function to compute the native structure of a protein. This provided the motivation to develop¹⁰ a theoretical approach to compute protein structure, first by making use of distance restraints and, subsequently, to rely on a physics-based potential function without the need to incorporate distance restraints. At about the same time, Anfinsen¹¹ identified spontaneous protein folding, and introduced the thermodynamic hypothesis for a theoretical approach, and we expanded our interest from determining structure to also determining folding pathways (first by experiment, and later by theory).

5 Experimental Studies of Oxidative Folding of RNase A

Our experimental study of the oxidative folding of RNase A led to the mechanism shown in Figure 4. Figure 4(a) shows that a pre-equilibrium exists between the unfolded forms

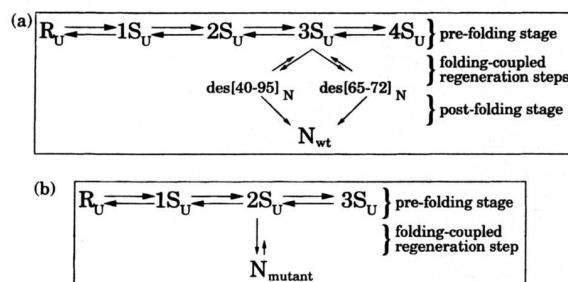


Figure 4. (a) Oxidative folding of wild-type RNase A. (b) Oxidative folding of two three-disulfide mutants of RNase A ($C40A/C95A$ and $C65S/C72S$).

of reduced RNase A, R , and various ensembles of disulfide-bonded intermediates¹². The rate-determining step¹³ is the reshuffling of the three-disulfide ensemble, $3S$, by SH/SS interchange, to form two main intermediates, $des[40-95]_N$ and $des[65-72]_N$ which each contain three native disulfide bonds but lack the 40-95 and 64-72 disulfide bonds, respectively. These two native intermediates^{14, 15} rapidly form the native structure of the wild-type protein. As shown in Figure 4(b), two very minor pathways exist^{16, 17} in which the $2S$ ensemble undergoes oxidation to $des[40-95]_N$ and $des[65-72]_N$, which could be detected only with the aid of mutants which lacked the 40-95 and 65-72 disulfide bonds, respectively.

The overall scheme for the oxidative folding of RNase A is shown in Figure 5. Of the 28 possible $1S$ species, 40% have the native 65-72 disulfide bond, and 10% have the non-native 58-65 disulfide bond, and the remaining 26 species accumulate only to the extent of $<10\%$ each in folding of the whole protein¹⁸. The 65-72 disulfide bond persists increasingly in the remainder of the pathway¹⁹ to form $des[40-95]_N$. Interestingly, the same 40:10 ratio that is found in the protein is also found when a fragment of reduced RNase A from Cys 58 to Cys 72 is oxidized^{20, 21}. This result is attributed to preferential native-forming interactions²² and not to entropic effects in the 65-72 loop, since both possible loops (58-65 and 65-72) have the same size; it is this kind of physics that is revealed by such experimental studies, and by our concomitantly developed molecular mechanics approach.

6 All-atom Determination of Protein Structure and Folding Pathways

Progressing from our initial work¹⁰ in 1965, with a hard-sphere potential, we developed an all-atom ECEPP (Emperical Conformational Energy Program for Peptides) force field²³,

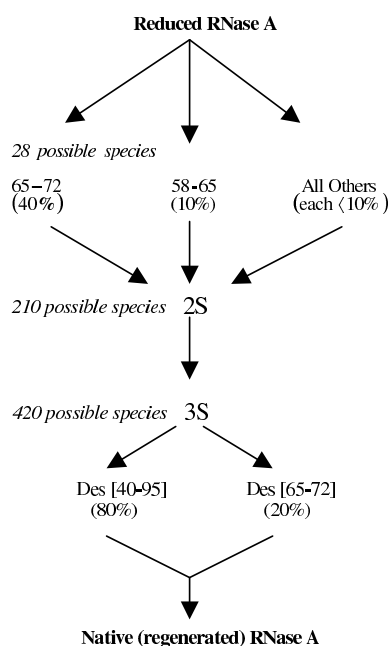


Figure 5. Overall scheme of oxidative folding of RNase A

and improved it in subsequent years, the latest version of which²⁴ appeared in 2006. The ECEPP force field and various procedures that we developed for global optimization of the potential energy were described in a recent review²⁵. The largest protein whose structure we have computed with the all-atom force field is the 46-residue three-helix bundle, protein A²⁶, and the 36-residue villin headpiece²⁷.

Our initial attempt²⁸ to compute an all-atom folding pathway made use of the stochastic difference equation method of Elber²⁹. The pathways were computed from each of a large ensemble of unfolded states of protein A to the final folded state, and then averaged. It was found that the C-terminal helix folded first, followed by the N-terminal helix, and then the middle helix. Various folding pathways have been proposed for protein A, and it has been found³⁰ that environmental factors and different components in the various force fields used may account for the reported differences.

7 A Hierarchical Approach to Protein Structure and Folding Pathway Prediction

In order to compute protein structures larger than those of protein A, we have developed a hierarchical procedure which initially makes use of a united-residue (UNRES) model of a polypeptide chain³¹⁻³⁷ together with a conformational space annealing (CSA) procedure³⁸ to search the UNRES conformational space to find the *region* in which the global minimum might lie. Then the lowest-energy structures are converted from the UNRES representation

to an all-atom one^{39,40} whose ECEPP energy (including hydration) can then be globally optimized.

The UNRES model consists of virtual-bond chains for the backbone and side chains, with the less-important degrees of freedom (rotation of the peptide groups around their virtual $C^\alpha-C^\alpha$ bonds, internal rotations about side-chain bonds, etc.) averaged out. The force centers are the positions of the averaged-out peptide groups and the ends of the virtual bonds at the center of gravity of the side chains. The UNRES energy consists of interactions between these force centers, the energies to vary the positions and rotational states of side chains, the energies to vary the angle between successive backbone virtual bonds, the torsional angles around the backbone virtual bonds, and double torsions around two neighboring virtual bonds, and multi-body interactions. The CSA procedure is essentially a genetic algorithm in which a finite set of widely- dispersed UNRES minima are forced to coalesce to the region of the global minimum.

Performance in successive blind tests from CASP3 to CASP7 has provided sufficient confidence to encourage us to develop^{41,42} and apply⁴³⁻⁴⁵ a molecular dynamics treatment based on UNRES. Our recent work with this molecular dynamics approach is being discussed at this workshop by A. Liwo⁴⁶.

8 Conclusions

The evolution of the development of our experimental and theoretical approaches to gain an understanding of the fundamental physics that controls protein structure and folding pathways has been traced. It is elaborated upon in the accompanying article by Liwo et al⁴⁶. Current work is focused on the use of the molecular dynamics approach with UNRES, and the improvement of this methodology including introduction of entropic effects³⁷.

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